Historic, archived document

Do not assume content reflects current scientific knowledge, policies, or practices.

LIBRARY
CURRENT SERIAL RECORD
MAY 29 1951
U.S. DEPARTMENT OF AGRICULTURE

1.932
A2Ag82

LIBRARY
RECEIVED
FEB 4 - 195?
U.S. DEPARTMENT OF AGRICULTURE

October, 1947.
Revisions: March, 1948,
February, 1950, October, 1950,
August, 1952.

AIC-168

ANNOTATED BIBLIOGRAPHY OF SUBTILIN: Assay, Microbiological Production, Purification and Chemistry, Bacteriology, Pharmacology, Therapeutic Activity, Food Preservation, and Related Compounds

J. C. Lewis
Western Regional Research Laboratory

Albany 6, California

Bureau of Agricultural and Industrial Chemistry
Agricultural Research Administration
UNITED STATES DEPARTMENT OF AGRICULTURE

ANNOTATED BIBLIOGRAPHY OF SUBTILIN

J. C. Lewis

Western Regional Research Laboratory
Albany, California

INTRODUCTION

The genus Bacillus has a long record of observed antibiotic activity. This bibliography covers reports of the antibiotic activity produced by a particular strain of Bacillus subtilis, first studied from this aspect in the Western Regional Research Laboratory, and of the antibacterial agent, subtilin, concentrated from cultures of this organism. This strain of B. subtilis was originally obtained from N. R. Smith of the U. S. Department of Agriculture, Bureau of Plant Industry, Soils, and Agricultural Engineering, under the number 231. It is now deposited in the American Type Culture Collection as number 6633 and in the culture collection of the Northern Regional Research Laboratory as B-543.

Subtilin has not found therapeutic use as yet, although its low toxicity may make it worthy of consideration in special applications where alternative agents are especially desired. In recent years subtilin has been studied for its possible value in control of food spoilage bacteria. Although practical processes are not available now, it seems reasonable that subtilin or other antibiotics may eventually have some utility in food preservation.

Subtilin has been prepared on a pilot-plant scale by several companies in the United States. It seems likely that subtilin could become available commercially if even a moderate usage for the compound were assured.

A discussion of the potency of various subtilin preparations is given below under ASSAY. Those articles originating from the Western Regional Research Laboratory are marked "WRRL" following the authors' names.

ASSAY

Most of the work on subtilin has been done with concentrates; crystalline subtilin has not yet been prepared. The most potent preparations made at the Western Regional Research Laboratory have appeared nearly homogeneous by electrophoresis, by fractional dialysis, and by fractional precipitation with sodium chloride (see Fevold et al., page 6. On the other hand, investigations at the research laboratories of Merck & Company, Rahway, New Jersey (see Brink et al., page 7, by countercurrent distribution, show that our most potent preparations are probably about 90 percent pure on the basis of the increased bacteriostatic potency of the principal fraction (referred to herein as "pure" subtilin). The arbitrary subtilin standard, lot L1263, referred to in some of the earlier papers from the WRRL, was 38.6 percent as potent as pure subtilin by turbidimetric assay with Micrococcus conglomeratus (see next below). Although some early preparations supplied from WRRL were as low as 20 percent pure, most of the preparations which have been distributed from WRRL were from 70 to 90 percent pute. Editorial insertions regarding potency of subtilin preparations used in various studies are indicated by brackets.

Sensitive organisms for the cup-plate assay of subtilin and subtilin esters are mentioned by Wilson *et al.*, page

Lewis, J. C., Humphreys, E. M., Thompson, P. A., Dimick, K. P., Benedict, R.G., Langlykke, A. F., and Lightbody, H. D., WRRL (authors Benedict and Langlykke, NRRL). The microbiological assay of subtilin. *Arch. Biochem.* 14(3):437-50, 1947. Subtilin was assayed by a turbidimetric method which depends on growth inhibition of *Micrococcus conglomeratus* (MY), *Streptococcus faecalis* (ATCC 7080). or *Staphylococcus aureus* (H). The cultures were incubated for 4 or 5 hours at 37°C. under nonsterile conditions. The antibiotic activity of crude culture extracts of *B. subtilis* (ATCC 6633) and of partially purified lots of subtilin could not be accounted for entirely in terms of a single active substance. Sixty to 70 percent ethanol was optimal for extraction of subtilin from crude cultures. The extraction was essentially complete in 15 minutes. Crude cultures lost substantial amounts of activity on steaming for 1 to 2 hours; the rate of destruction was faster at pH 6.4 to 7.1 than at 2.5. (Reprints exhausted.)

Housewright, R. D., Henry, R. J., and Berkman, S., Camp Detrick, Md. A microbiological method for the assay of subtilin. *Jour. Bact.* 55(4):545-50, 1948. A diffusion plate assay which utilizes filter paper discs and *Bacillus cereus* 247 is described. In the range of 15 to 32 mm., the zone diameter was linearly related to log subtilin concentration. Although precision was favored by high slope (doubling the concentration increased the zone diameter by 5.3 mm.), the method has low sensitivity. A 20 mm. zone required 200 ppm. of subtilin lot 118-T [45 percent as potent as pure subtilin by turbidimetric assay]. Increased zone diameter was favored by low pH in the range of 5.9 to 7.2, and by high NaCl concentration in the range of 0 to 2 percent.

Macmorine, H. G., and Slinn, G. S., Connaught Medical Research Laboratories, Univ. of Toronto. Plate techniques for the assay of subtilin. *Canad. J. Public Health* 39:203-08, 1948. Diffusion plate assays which utilize *Staphylococcus aureus* 209P are described. The diameter of the zones was increased by allowing a diffusion period before incubation during which the plates were refrigerated, and by incorporating 0.1 percent of Tween 80 in the seed layer. Filter paper discs were preferred to cups. The plate tests were only 1 to 2 percent as sensitive as turbidimetric tests, but were more reproducible.

MICROBIOLOGICAL PRODUCTION

Humfeld, H., and Feustel, I. C., WRRL. Utilization of asparagus juice in microbiological culture media. *Proc. Soc. Expt. Biol. and Med.* 54(2):232-35, 1943 *B. subtilis* (ATCC 6633) cultures grown for 24 hours at 35°C. on asparagus butt juice medium were adjusted to pH 2.5 and sterilized for 10 minutes at 10 lbs. of steam pressure. These preparations possessed marked antibiotic activity against *Staphylococcus aureus*, *Micrococcus conglomeratus*, *Lactobacillus casei*, and the plant pathogen, *Phytomonas michiganensis*. Less activity was found against the Gram-negative plant pathogens *Phytomonas juglandis* and *Erwinia amylovora*. (Reprints exhausted.)

Jansen, E. F., and Hirschmann, D. J., WRRL. Subtilin--An antibacterial product of *Bacillus subtilis*. Culturing conditions and properties. *Arch. Biochem.* *4(3):297-309, 1944.* *B. subtilis* (ATCC 6633) was grown in surface cultures on media containing sucrose, mineral salts, and several organic nitrogen sources including asparagine, glutamic acid, aspartic acid, casein, and tryptone. The addition of manganese (1 ppm.) proved essential for high antibiotic activity. Wide variations were noted in semi-quantitative serial dilution tests of antibacterial activity against *Staphylococcus aureus* (209), *Micrococcus conglomeratus* (MY), and *Lactobacillus casei* (ATCC 7049) of cultures acidified to pH 2.5 and sterilized for 10 minutes at 10 lbs. of steam pressure. The material having antibacterial activity was named *subtilin*, but was believed to consist of more than one substance. A crude concentrate was prepared by centrifuging and vacuum-drying cultures adjusted to pH 4.7. The active material was reported to be soluble in aqueous ethanol but not in 95 percent ethanol, to be diffusible, to be heat-labile under alkaline conditions but relatively stable at pH 2.5, and to be inactivated by light and by formaldehyde. (Reprints exhausted.)

Hassall, C. H., University Chemical Laboratories, Cambridge, England. Subtilin C: An antibiotic concentrate from *Bacillus subtilis*. *Nature* 161(4087):317-18, 1948. A note confirming that *B. subtilis* (ATCC 6633) produces an antibiotic active against Gram-positive bacteria and mycobacteria in moderate to high dilutions. A concentrate ("subtilin C") was prepared by autoclaving dried *B. subtilis* cells with 80 per cent aqueous alcohol, and collecting the precipitate formed by dilution of the extract and adjustment to pH 2.3. The active factor was slowly diffusible, light-sensitive, most stable at pH 2.5 but very labile in alkaline solution, inactivated by trypsin and pepsin, and gave positive tests for alpha-amino acids (ninhydrin), indole nuclei (Erlich), and phenol (Folin-Denis).

Anonymous, WRRL (announcements and brief abstracts of work done at WRRL on production, purification and assay of subtilin):
Production, concentration, properties, and assay of the antibiotic, subtilin. Mimeographed Circular AIC-106, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture. 1946.
Production of subtilin--A new antibiotic. *Drug and Cosmetic Industry* 60:478,1947.
Subtilin output and purification. *Oil, Paint, and Drug Reporter* 151:5, 55, 1947.
Subtilin from asparagus waste. *Chem. and Engin. News* 25:1070-71, 1947.

Lewis, J. C., Feeney, R. E., Garibaldi, J. A., Michener, H. D., Hirschmann, D. J., Traufler, D. H., Langlykke, A. F., Lightbody, H. D., Stubbs, J. J., and Humfeld, H., WRRL (authors Traufler and Langlykke, NRRL). Subtilin production in surface cultures. *Arch. Biochem.* 14(3):415-25, 1947. The highest yields of subtilin in shallow-layer *cultures* (as determined by the antibiotic activity of aqueous ethanol extracts) were obtained with a beet molasses medium, which required the addition of $(NH_4)_2HPO_4$ (0.8 percent) and manganese (50 ppm.). [The maximum yield reported is equivalent to 770 mg. of pure subtilin per liter of culture.] Good yields were obtained on media prepared from asparagus butt waste press juice, molasses and grain worts, and corn steep liquor. An incubator temperature of 35°C., with peak temperatures in the cultures 4° to 8°C. higher, was approximately optimal. Maximum yields were usually attained in cultures 1 to 2 cm. deep after 24 to 48 hours. Prolonged incubation of asparagus and beet molasses media resulted in a drop in antibiotic activity. The distribution of subtilin between pellicle and culture filtrate varied widely for different media. Two other strains of *B. subtilis* did not produce appreciable amounts of subtilin. (Reprints exhausted.)

Stubbs, J. J., Feeney, R. E., Lewis, J. C., Feustel, I. C., Lightbody, H. D., and Garibaldi, J. A., WRRL. Subtilin production in submerged culture. *Arch. Biochem.* 14(3):427-35, 1947. Excellent yields of subtilin were produced in fermenters of one-liter capacity equipped with high-speed stirrers for dispersion of air. Asparagus-juice concentrate, when diluted to 14 percent solids, steamed for 30 minutes, adjusted to pH 7.0, inoculated, and incubated at 35°C. under aeration, at a rate of one volume of air per minute for 10 hours, gave the highest yield [equivalent to 700 mg. of pure subtilin per liter of culture]. Subtilin was also produced in high yield but at a slower rate on media prepared from beet molasses. (Reprints exhausted.)

Stubbs, J. J., Feeney, R. E., Garibaldi, J. A., and Feustel, I. C., WRRL. Process of producing subtilin. U. S. Patent 2,524,089, Oct. 3, 1950. This patent covers the production of subtilin under submerged conditions as described by Stubbs *et al.*, cited above.

Feeney, R. E., Lightbody, H. D., and Garibaldi, J. A., WRRL. Zinc as an essential element for growth and subtilin formation by *Bacillus subtilis*, *Arch. Biochem.* 15(1):13-17, 1947. Zinc was found essential for growth and subtilin formation by *B. subtilis* (ATCC 6633) in shallow-layer stationary cultures. A zinc-free simplified medium was prepared (by extraction with diphenylthiocarbazone in carbon tetrachloride) which permitted only slight growth and subtilin yields [less than 8 mg. of pure subtilin per liter]. This medium gave a linearly increasing response in subtilin yields when 0.03 to 0.5-1.0 ppm. of zinc was added. Maximum yields were obtained over the range of 1 to 10 ppm. of added zinc. A partial zinc deficiency was also observed on an unpurified medium. This appears to be the first demonstration of an absolute requirement for zinc in bacterial growth. Cadmium, the only element capable of substitution for zinc, was only partially effective. (Reprints exhausted.) (Preliminary announcement in *Fed. Amer. Socs. Expt. Biol. Proc.* 6(1):250, 1947.)

Feeney, R. E., Garibaldi, J. A., and Humphreys, E. M., WRRL. Nutritional studies on subtilin formation by *Bacillus subtilis*. *Arch. Biochem.* 17(3):435-45, 1948. The nutritional requirements for the production of relatively high levels of subtilin in shallow-layer cultures were restricted to an appropriate source of carbohydrate or related compound, inorganic sources of N, P, and S, and mineral elements. NH_3 and NO_3^- were equally satisfactory, while organic sources of N were not superior to inorganic sources. Approximate quantitative requirements were: sucrose, 5 to 7 percent; N, 2000 ppm., P, 150 ppm., and S, 80 ppm. The following simplified medium based on this study gave yields in the range of 580 to 770 mg. of subtilin [recalculated equivalent of pure subtilin] per liter of culture under submerged conditions as described previously by Stubbs et al. (page 4 : sucrose, 10; citric acid, 1.2; Na_2SO_4, 0.4; yeast extract, 0.5; $(NH_4)_2HPO_4$, 0.4 percent;

chlorides to give K, 400; Mg, 50; Zn, 5; Fe, 5; and Mn, 5 ppm.; and NH_4OH to pH 6.8-6.9. (Preliminary announcement in Fed. Amer. Socs. Expt. Biol. Proc. 6(1): 250, 1947.)

Feeney, R. E., and Garibaldi, J. A., WRRL. Method of preparing subtilin. U.S. Patent 2,508,378, May 23, 1950. This patent covers the conditions of mineral nutrition described by Feeney et al., cited above.

Feeney, R. E., and Garibaldi, J. A., WRRL. Studies on the mineral nutrition of the subtilin-producing strain of Bacillus subtilis. Arch. Biochem. 17(3):447-58, 1948. The mineral requirements to produce 10 gm. of dry cells per liter of medium containing sucrose, ammonium nitrogen, asparagine, and glutamic acid were found to be approximately as follows (in ppm.): K, 125; Mg, 2.5; Fe, 1.2; Mn, 0.7; and Zn, 0.5. The requirements for subtilin formation were generally higher than the growth requirements. Rb replaced K for growth but not for subtilin production. Except for this and a partial replacement of Zn by Cd, the requirements for the above elements were specific. Ca had a detrimental effect on subtilin production, while citrate generally had a beneficial effect.

Humfeld, H., WRRL. An improved laboratory fermentor for submerged culture investigations. Jour. Bact. 54(6):689-96, 1947. A newly designed laboratory fermentor of 15 to 20 liters capacity, which gives improved air dispersion and is equipped with a mechanical foam breaker, gave a good subtilin fermentation.

Humfeld, H., Aeschlimann, E., and Hoffman, J. R., WRRL. Fermentor for submerged culture. U.S. Patent 2,542,031, Feb. 20, 1951. This patent covers the fermentor described by Humfeld, cited above.

Garibaldi, J. A., and Feeney, R. E., WRRL. Subtilin production. Indus. and Engin. Chem. 41(2):432-35, 1949. Production on a 150 to 200 liter scale with asparagus juice, beet molasses, and simplified media is described. Maximum yields of 460 mg. of subtilin /recalculated equivalent of pure subtilin/ per liter of medium were obtained with the sucrose-yeast extract-citrate-salts medium published previously by Feeney et al. (page 4). The fermentations were conducted at 35°C. with aeration at the rate of 1 volume of air per volume of medium per minute. Maximum antibiotic potency was obtained in 11 to 12 hours.

PURIFICATION AND CHEMISTRY

Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., WRRL. Purification and some properties of subtilin. Arch. Biochem. 15(1):1-11, 1947. Subtilin concentrate was obtained by extraction with 70 percent ethanol from pellicles produced by B. subtilis (ATCC 6633) on asparagus butt juice medium. The extract was concentrated in vacuo to remove ethanol, whereupon the active material precipitated. To remove inactive contaminants the dried filter cake was extracted successively with 95 percent ethanol, and with 85 percent ethanol containing 1 percent each of acetic acid and NaCl. The active material was then obtained in solution by extracting the residue with 0.16M acetate at pH 4.6. The extracts were treated with filtering aid, deionized with exchange resins, concentrated, and lyophilized to yield a dull white powder. The subtilin preparation was soluble to at least 10 percent in salt-free acidified water, but to less than

0.5 percent at pH 6 to 9. In the presence of 0.5 percent NaCl at acid pH the solubility was less than 0.5 percent. The preparation was soluble in 0 to 80 percent ethanol, and in methanol, but not in dry ethanol, dry butanol, dry amyl alcohol, acetone, ether, petroleum ether, or chloroform. It was about 0.5 percent soluble in *n*-butanol saturated with water. It diffused fairly rapidly through cellophane. The preparation contained 15.8 percent total (Kjeldahl) nitrogen. The amino nitrogen content of 1.7 percent increased to about 12.6 percent after acid hydrolysis. It contained 4.2 percent sulfur and a trace of phosphorus. It was levorotatory. Subtilin was inactivated by alkali, and the activity decreased on incubation with pepsin and trypsin. Dry subtilin preparations lost activity when stored for a period of months at room temperature, but dilute solutions at pH 2.5 appeared stable when stored in the refrigerator. The butanol extraction method of Fevold *et al.*, cited below, was noted briefly. (Reprints exhausted.)

Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., WRRL. Process for extraction and purification of subtilin. U. S. Patent 2,459,139, Jan. 18, 1949. This patent covers the process of Dimick *et al.*, cited above.

Fevold, H. L., Dimick, K. P., and Klose, A. A., WRRL. Isolation of subtilin from submerged cultures. *Arch. Biochem.* 18(1):27-34, 1949. A method for the isolation of subtilin is described which depends on the extraction of the culture broth at pH 2 with one-half volume of *n*-butanol, and separation of the two phases in a Sharples centrifuge. Subtilin was recovered from the butanol phase (a) by addition of one-half volume of petroleum ether to the butanol extract and extraction with 1 percent acetic acid followed by precipitation of subtilin from the aqueous extract with 10 percent NaCl, or (b) by addition of NaCl as described by Lineweaver *et al.*, cited below, followed by recovery of the precipitate in the Sharples centrifuge. The moist cake of crude subtilin was extracted twice with 10 volumes of absolute ethanol. Toxic impurities remaining were removed by dissolving to give a 0.5 to 0.75 percent solution of the extracted cake at pH 3.0 to 3.5, adding NaCl to 0.4 percent, and adjusting to pH 4.6 with 0.1 percent NaOH. The flocculent precipitate was removed and re-extracted twice in the same fashion. Subtilin was recovered from the combined extracts by precipitation with 10 percent NaCl, removal of excess NaCl by washing with 1 percent NaCl and then with absolute ethanol, and drying. About 50 percent over-all yields were obtained. The product appeared to be essentially homogeneous by fractional dialysis, salt fractionation, and electrophoretic studies. The LD_{50} for subcutaneous injection in mice was about 3 gm. per kg. of body weight. [The maximum potency obtained was about 90 percent of that of pure subtilin.] (Preliminary announcement in *Fed. Amer. Soc. Expt. Biol. Proc.* 6(1):247-48, 1947.)

Dimick, K. P., Stubbs, J. J., Garibaldi, J. A., Lightbody, H. D., and Fevold, H. L., WRRL. Purification of subtilin. U. S. Patent 2,476,085, July 12, 1949. This patent covers the process of Fevold *et al.*, cited above.

Lineweaver, H., Klose, A. A., and Alderton, G., WRRL. An alternate step for the isolation of subtilin. *Arch. Biochem.* 16(2):311-13, 1948. Subtilin was salted out of butanol extracts of *B. subtilis* (ATCC 6633) culture by adding 60 gm. of NaCl per liter of butanol and adjusting the pH of the aqueous phase to 5. The precipitate was collected in the bowl of a Sharples continuous centrifuge which was adjusted to remove separately the dehydrated butanol and the salt-water. Relatively little subtilin was lost in these liquid phases.

Lineweaver, H., Klose, A. A., and Alderton, G., WRRL. Technique for isolating subtilin. U. S. Patent 2,481,763, Sept. 13, 1949. This patent covers the process of Lineweaver *et al.*, cited above.

Brink, N. G., Mayfield, J., and Folkers, K. Research Laboratories of Merck & Co., Rahway, N.J. The purification of subtilin concentrates by counter-current distribution. *Jour. Amer. Chem. Soc.* 73(1):330-31, 1951. Subtilin of the highest potency isolated as described by Fevold *et al.* (1949) (see page 6 of this bibliography) was distributed into 12 to 24 fractions between *n*-butanol and water or sec-butanol and water. A small amount of material of low bacteriostatic potency was separated, while the potency of the most active fractions was increased by approximately 10 percent over that of the starting material. Some physical and chemical constants for the most active fractions are reported.

Carson, J. F., Jansen, E. F., and Lewis, J. C., WRRL. Esterification of subtilin and its effect on solubility and *in vitro* bacteriostatic activity. *Jour. Amer. Chem. Soc.* 71(7):2318-22, 1949. The preparation of methyl, ethyl, hydroxyethyl, and hydroxypropyl esters of subtilin is described. Esterification with methanol in the presence of 0.03*N* HCl at 0°C. is specific for carboxyl groups, a maximum of 6 to 7 equivalents of ester being introduced per 10^4 gm. of subtilin. These esters have approximately two times the solubility of subtilin in 0.9 percent NaCl at pH 7.3 and approximately two and four times the bacteriostatic activity of subtilin for *Streptococcus faecalis* (ATCC 7080) and *Micrococcus conglomeratus* (MY). When the esterification was carried out at 25°C. amide nitrogen decreased and as much as 14 equivalents of ester were introduced per 10^4 gm. of subtilin. With 9 to 11 equivalents the solubility in 0.9 percent NaCl at pH 7.3 increased 30- to 50-fold to 3 to 5 percent. Certain hydroxyethyl esters were characterized by increased bacteriostatic activity or by greatly increased solubility. Ethyl and hydroxypropyl esters did not show increased bacteriostatic activity, and were characterized by only slight or moderate increases in solubility in physiological salt solution. (Preliminary announcement in *Fed. Amer. Socs. Expt. Biol. Proc.* 6(1):270, 1947.)

Lewis, J. C., and Jansen, E. F., WRRL. Alcohol derivatives of subtilin. U. S. Patent 2,495,743, Jan. 31, 1950. This patent covers the subtilin esters containing up to 7 equivalents of methoxyl groups per 10^4 gm. of subtilin, as described by Carson *et al.*, cited above.

Carson, J. F., and Lewis, J. C., WRRL. Methyl esters of subtilin. U. S. Patent 2,520,908, Sept. 5, 1950. This patent covers the highly soluble subtilin esters containing more than 7 equivalents of methoxyl groups per 10^4 gm. of subtilin as described by Carson *et al.*, cited above.

Gore, R. C., and Peterson, E. M., American Cyanamid Co., Stamford, Conn. Infrared studies. *Ann. N.Y. Acad. Sci.* 51(5):924-34, 1949. The infrared spectrum of subtilin, among other antibiotics, is presented (without comment).

Alderton, G., and Fevold, H. L., WRRL. Lanthionine in subtilin. *Jour. Amer. Chem. Soc.* 73(1):463-64, 1951. Meso-lanthionine was isolated from hydrochloric acid hydrolyzates of subtilin in yield equivalent to approximately 10 percent of the sulfur. It was found identical with meso-lanthionine isolated from alkali-treated hair, by X-ray powder photographs, by degradation of the benzoyl derivative with Raney nickel, and through the benzoyl and carbobenzoxy derivatives.

Stansly, P. G., and Ananenko, N. H., American Cyanamid Co., Stamford, Conn. Resistance of polymyxin to some proteolytic enzymes. *Arch. Biochem.* 15(3):473-74, 1947. Unlike polymyxin, subtilin was largely inactivated by pepsin (pH 2.2-4.8), trypsin (pH 4.4-7.5), pancreatin (pH 4.4-7.5), and erepsin (pH 6.1-7.8).

Owens, H. S., and Maclay, W. D., WRRL. Subtilin-pectin derivative. U. S. Patent 2,555,364, June 5, 1951. This patent covers mixtures of subtilin and pectin which are characterized by approximately 50-fold greater solubility than subtilin alone under physiological conditions.

Lewis, J. C., and Snell, N. S., WRRL. The amino acid composition of subtilin. *Jour. Amer. Chem. Soc.* 73(10):4812-16, 1951. The solids and nitrogen of subtilin were accounted for almost quantitatively by alanine 2,3,L-aspartic acid 4.1, L.glutamic acid 12.2, glycine 4.7, L-isoleucine 4.0, L-leucine 15.5, L-lysine 12.3, lanthionin 6.4, L-phenylalanine 4.8, L-proline 3.3, tryptophan 5.1, L-valine 3.3, a yet unidentified sulfur-containing amino acid 27.5 and amide nitrogen 2.2. The figures indicate the content as g. in the hydrolyzate from 100 g. of subtilin free base (16.3 percent N). Seven of the amino acids usually found in proteins (arginine, cystine (or cysteine), histidine, methionine, serine, threonine, and tyrosine) were virtually absent (0.1 percent or less). The empirical formula deduced from the analytical data is $\mathrm{Gly_2Ala_1Val_1Leu_4Ileu_1Pro_1Phe_1Try_1Lys_3Asp_1Glu_3Lan_1(C_7H_{12}O_3N_2S)_4(amide)_5(H_2O)_1}$, corresponding to a molecular weight of 3188. This forumla accounts for 38 of the 40 nitrogen equivalents determined per 3420 g., and for a somewhat lower proportion of the solids. This simple formula cannot be complete, since the numbers of free amino and carboxyl groups found experimentally are significantly fewer than those calculated from the empirical formula.

Carson, J. F., WRRL. The free amino groups of subtilin. *Jour. Amer. Chem. Soc.* 74(6):1480-83, 1952. Deamination of subtilin with nitrous acid, followed by hydrolysis and microbiological assay, and reaction of subtilin with dinitrofluorobenzene, followed by hydrolysis and chromatography of the DNP-amino acids, showed that the free amino groups of subtilin are contributed by lysine and by the two sulfur diamino dicarboxylic acids. Each lysine has the alpha-amino group linked and the epsilon-amino group free. The two sulfur acids cannot exist in subtilin with both amino groups free, but must have one or neither amino group free.

BACTERIOLOGY

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--An antibiotic produced by *Bacillus subtilis*. I. Action on various organisms. *Proc Soc. Expt. Biol. and Med.* 60:60-64, 1945. Partially purified subtilin was found to inhibit growth of a number of Gram-positive bacteria, the Gram-negative *Neisseria catarrhalis* and *N. gonorrhoeae*, certain acid fast bacteria including *Mycobacterium tuberculosis*, and certain pathogenic fungi. The effect on *M. tuberculosis* was bacteriostatic at low and bactericidal at higher concentrations.

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Studies on subtilin fastness *in vitro*. *Jour*. *Bact*. 55(4):463-69, 1948. *Staphylococcus aureus* became resistant to 400 times the concentration of subtilin initially bacteriostatic (2.5 ppm. of partially purified subtilin) after 6 serial transfers in liquid medium containing increasing concentrations of subtilin. Similarly *Mycobacterium phlei*, initially sensitive to 2.5 ppm., became resistant to 500 ppm. after 9 transfers, and *Escherichia communior*, initially sensitive to 300 ppm., became resistant to 1000 ppm. after 3 transfers. The resistant strains tended to retain much of their resistance for as many as 75 daily transfers in liquid medium free of subtilin.

Wong, S. C., Hambly, A. S., and Anderson, H. H., University of California Medical School, San Francisco. Use of modified Dubos and Davis medium for demonstration of antibiotic activity of subtilin against *Mycobacterium tuberculosis*. *Jour*. *Lab*. *Clin*. *Med*. 32(7):837-41, 1947. In Dubos medium partially purified subtilin was bacteriostatic at 1/400,000 dilution, and bactericidal only at 1/20,000 or lower dilution. A virulent strain of *M*. *tuberculosis* was used.

Anderson, H. H., and Chin, Y. C., University of California Medical School, San Francisco. Antibiotic activity of subtilin and streptomycin in the presence of BAL. *Science* 106(2765):643-44, 1947. BAL (2,3-dimercaptopropanol) at a dilution of 1/100,000 in Dubos medium increased the bacteriostatic potency of subtilin against 3 pathogenic and 1 non-pathogenic strains of *Mycobacterium tuberculosis* by 2 to 16 times, and of streptomycin to a lesser degree.

Anderson, H. H., University of California Medical School, San Francisco. New surface active antibiotics. *Jour*. *Invest*. *Dermatology* 8(1):25-33, 1947. Certain wetting agents, "Tergitol" and "Tween 80", substantially increased the bacteriostatic action of subtilin against *Lactobacillus plantarum*. Sodium lauryl sulfate, on the other hand, greatly reduced the activity of subtilin. Serum albumin (5 percent) or globulin (1 percent) did not repress the activity of subtilin. In Dubos medium with 0.05 percent of Tween 80, partially purified subtilin inhibited growth of *Mycobacterium tuberculosis* for 12 days at 1/400,000 dilution. Subtilin ruptured the cell membrane of *Endamoeba histolytica*, *in vitro*.

Anderson, H. H., Johnstone, H. G., and Hansen, E. L., University of California Medical School, San Francisco. Experimental chemotherapy of amebiasis. *Amer*. *Jour*. *Tropical Med*. 27:153-160, 1947. *Endamoeba histolytica* was hilled *in vitro* by cytolytic concentrations of subtilin. In egg slope medium, approximately 50 ppm. was required, in liquid liver medium approximately 2 ppm. of partially purified subtilin sufficed.

Chin, Y. C., University of California Medical School, San Francisco. Effect of some derivatives of subtilin on tubercle bacilli and rabbit leukocytes *in vitro*. *Fed*. *Amer*. *Socs*. *Expt*. *Biol*. *Proc*. 7(1):211-12, 1948. The tuberculostatic activity of methylated, ethylated, and glycolated derivatives of subtilin against *Mycobacterium tuberculosos* ($H37R_V$) in Dubos medium was either no different from subtilin or was increased 2-fold. The low toxicity toward rabbit leukocytes was unchanged, except for a 3-fold increase for one sample.

Dubos, R. J., Rockefeller Institute. The effect of wetting agents on the growth and susceptibility of tubercle bacilli. *Proc. 48th General Meeting Society of American Bacteriologists*, May 1948, pp. 83-84. Certain arylalkyl polyethers of phenols wet the surfaces of tubercle bacilli without affecting their metabolism as do the water-dispersible esters of oleic, stearic, and palmitic acids (Tweens) which are more readily hydrolyzed by tissue enzymes. All surface-active substances tested which promoted dispersed growth also increased the susceptibility to a wide variety of antibacterial agents. For example, strains of mycobacteria which grew in oleic acid albumin medium in the presence of 100 ppm. of partially purified subtilin were inhibited by 5 ppm. in media containing the proper wetting agent.

Knight V., and Tompsett, R., Cornell University Medical College. Relationship of type of growth of *M. tuberculosis* to antituberculous activity of subtilin. *Proc. Soc. Expt. Biol. and Med.* 73:55-60, 1950. Subtilin was found to be highly inhibitory to pneumococci, streptococci, and tubercle bacilli *in vitro*, and to have a high therapeutic activity for infections of the first two organisms but not for tuberculosis in mice. It was found that subtilin inhibited the tubercle bacillus only when dispersed growth was obtained (by addition of non-ionic wetting agents (Tween)). The inhibitive concentration of (80 percent pure) subtilin in the presence of Tween was 3 to 12 ppm. Serum antagonized the action of the wetting agent and permitted clumped growth. Under this condition subtilin did not inhibit growth at 100 ppm. When sufficient wetting agent was used antitubercular activity was demonstrated in serum from subtilin-treated rabbits. (Preliminary announcements in *Jour. Clin. Invest.* 27:544, 1948; *Proc. 48th Gen. Meeting Society of American Bacteriologists*, May 1948, p. 84.)

Kraft, M. E., and Spencer, G. R., University of Wisconsin. *In vitro* effect of certain antibacterial agents on organisms encountered in bovine mastitis. *Proc. Soc. Expt. Biol. and Med.* 70:176-79, 1949. Subtilin [80 percent pure] killed 3 Gram-positive bacteria (*S. aureus*, *Str. agalactiae*, and *C. pyogenes*) associated with bovine mastitis at a dilution of 1:80,000, and inhibited growth at twice this dilution. Gram-negative bacteria were not affected.

Goodman, J. J., and Henry, A. W., University of Alberta, Edmonton, Canada. Action of subtilin in reducing infection by a seed-borne pathogen. *Science* 105:320-21, 1947. Barley seeds exposed simultaneously to a 0.1 percent solution of partially purified subtilin and *Xanthomonas translucens cerealis* for 24 hours did not yield infected seedlings, but with 0.02 percent subtilin infection approached that of controls without subtilin. Only partial protection was obtained when the seeds were infected, dried, and subsequently treated with subtilin.

Katznelson, H., and Hood, E. G., Central Experimental Farm, Ottawa, Canada. Influence of penicillin and other antibiotics on lactic streptococci in starter cultures used in cheddar cheese-making. *Jour. Dairy Sci.* 32(11):961-68, 1949. Subtilin inhibited these organisms in the range from 0.01-1 ppm; only penicillin of 5 other antibiotics was more active.

Katznelson, H., Dept. of Agriculture, Ottawa, Canada. The influence of antibiotics and sulfa drugs on *Bacillus larvae*, cause of American foulbrood of the honey bee, *in vitro* and *in vivo*. *Jour. Bact.* 59(4):471-79, 1950. Subtilin proved relatively

Hart, P. D'a., and Moss, B., National Institute for Medical Research, Mill Hill, London, N.W. 7. The distinction of licheniformin from subtilin by cross-reactions with antibiotic-resistant strains of *Mycobacterium phlei*, *Jour. Gen. Microb.* 4: 244-56, 1950. Licheniformin and subtilin induced resistance in *Mycobacterium phlei* more slowly and to a much smaller degree than did streptomycin. Strains resistant to licheniformin and subtilin tended to revert, whereas streptomycin-resistance was stable. Licheniformin resistance was somewhat more difficult to induce and less stable than subtilin resistance. Cross-resistance tests of bacteriostatic activity between the resistant strains and the three antibiotics distinguished completely licheniformin from both subtilin and streptomycin. Although the difference between licheniformin and subtilin was largely confirmed by a more sensitive bactericidal test, with viable counts after varying periods of exposure of the resistant strains to the antibiotics in nutrient medium, the test, nevertheless, revealed a minor overlap between the two antibiotics, which was either the result of contamination of one by the other or, more probably, the expression of some common chemical property. Licheniformin and subtilin differed in the speed of their lethal action on *M. phlei*, subtilin being much the slower.

Asher, T. M. The two opposing effects of subtilin on bacteria. *Jour. Gen. Microb.* 4:xvi, 1950. Subtilin inhibited growth and respiration of susceptible bacteria, and lysed the cells at a particular phase of the growth cycle. (Abstract.)

Salle, A. J., University of California at Los Angeles. The inactivation of influenza and Newcastle disease viruses by subtilin. *Proc. 50th General Meeting Society of American Bacteriologists*, May, 1950, p. 72. Subtilin inactivated the viruses of influenza A (PR8) and Newcastle disease (Calif. strain 64792) when tested in 10-day embryonated eggs. Three mg. of subtilin per egg gave optimal results.

Jann, G. J., UCLA, Los Angeles. A study of the factors influencing subtilin activity by the serial dilution and manometric technics. *Proc. 50th General Meeting of the Society of American Bacteriologists*, May, 1950, p. 97-98. Various reducing agents, urea, or horse serum failed to reduce the activity of subtilin.

Levaditi, C., and Henry, J., Institut Alfred-Fournier, Paris. La subtiline-mecanisme de son activite antibiotique. *Presse Medicale* 56:493-94, 1948. (Also reprinted in Levaditi, C., *et al.*, "Antibiotiques d'Origine Fougique, Bacterienne on Vegetale", J.-B. Balliere et Fils, Paris.) In relatively high concentrations subtilin lysed *Trypanosoma equiperdum*, *Treponema pallidum*, and *Spirochaeta duttoni*. Changes in morphology and staining behavior were induced by subtilin in *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus anthracis*. The changes were less marked than those induced by penicillin or streptomycin, and required concentrations of subtilin greater than those required to inhibit growth.

Kaiser, E., Instituto Sieroterapico Milanese, Serafino Belfanti, Über die Wirkung von Subtilin and Subtilin-Tyrothricin-Kombinationen *in vivo* et *in vitro*. *Weiner Med. Wochenschrift* 101(14):254-57, 1951. Subtilin and tyrothricin individually inhibited the respiration of *Staphylococcus aureus*, and had a synergistic action in combination, which was expressed both in the extent and the irreversibility of the inhibition. Synergistic effects were also noted in the prevention of localized infections of *S. aureus*, *Streptococcus haemolyticus*, and *Corynebacterium diphtheriae* in mice.

Jann, G. J., Streitfeld, M. M., and Salle, A. J., University of California at Los Angeles. Fastness of *Micrococcus pyogenes* var. *aureus* to subtilin and to combinations of subtilin and streptomycin. Jour. Bact. 63(3):353-62, 1952. The virulent Smith strain of *M. pyogenes* var. *aureus* became resistant to 50 times the concentration of subtilin initially bacteriostatic after only two transfers to increasing concentrations of subtilin. The resistance was retained well on long continued daily transfers in the absence of subtilin and was accompanied by a loss of virulence for mice. Enhanced resistance to subtilin, streptomycin, or both, was demonstrated. Subtilin and streptomycin were inhibitive in lower concentrations in mixtures than separately. Subcultures from such mixtures displayed enhanced resistance to both subtilin and streptomycin. Gelatinase activity was absent for the subtilin-streptomycin resistant variants, intermediate for the original strain, and increased for the subtilin-resistant streptomycin-sensitive variants. (Preliminary announcement in *Proc. 52nd General Meeting of the Society of American Bacteriologists*, May, 1952, p. 58.)

Sacks, L. E., WRRL. Subtilin considered as a germicidal surface-active agent. *Antibiotics and Chemotherapy* 2(2):79-85, 1952. Subtilin induced a rapid and permanent reduction in the metabolic activity of *Micrococcus pyogenes* var. *aureus* and *M. conglomeratus* on a variety of substrates, a rapid drop in the viable count, and a rapid leakage of phosphorus and nitrogen-containing cell constituents. These properties are characteristic of the action of tyrocidine and the germicidal surface-active agents. (Preliminary announcement in *Proc. 51st. General Meeting of American Bacteriologists*, May, 1951, pp. 69-70.)

Williams, O. B., and Campbell, L. L., Univ. of Texas, Austin. The effect of subtilin on thermophilic flat sour bacteria. *Food Research* 16(4):347-52, 1951. All of 10 strains of facultative thermophilic flat sour bacteria were inhibited for 40 days by concentrations of subtilin [78 percent pure] ranging from 3 to 15 mg. per liter, and all of 20 strains of the obligate type were inhibited similarly by 10 to 50 mg. per liter. Relatively few were inhibited more by being heated in the presence of subtilin at 100°C. for 5 minutes. Periodic plate counts on 4 of each of the two types of thermophiles were made. No effect of subtilin was observed with the obligate thermophiles, whereas the numbers of facultative thermophiles were greatly reduced for 3 of 4 strains by heating in the presence of approximately the minimal inhibitive concentration of subtilin for growth. However, in these cases a few individuals were noted to survive from the original inoculum of approximately 30,000 spores. A substantial proportion of survivors were likewise noted when an inoculum of 500,000 spores was heated in the presence of 50 mg. of subtilin per liter. (Abstract in Bact. Proc, 1951, p. 16.)

Williams, O. B., and Fleming, T. C., Univ. of Texas, Austin. Subtilin and the spores of Clostridium botulinum. Antibiotics and Chemotherapy 2(2):75-78, 1952. Spores of five strains of Cl. botulinum were inhibited from germination after heating 10 or 20 minutes at 98°C. in the presence of 5 to 20 ppm. of partially purified subtilin when 10 spores were added per tube and by 20 to 120 ppm. when 100,000 spores were used. The tubes were observed for 60 days, and it was noted that outgrowth was delayed longer with the higher concentrations of subtilin. Incubating high densities of spores in medium with 120 ppm. of subtilin for 3 days, when followed by centrifuging and washing, had no adverse effect on spore germination as judged by rapidity of germination and by quantitative counts.

LeBlanc, F. R., Devlin, K. A., and Stumbo, C. R., Washington State College, Pullman, Wash. Antibiotics in food preservation. I. The influence of subtilin on the thermal resistance of spores of Clostridium botulinum and P.A. 3679. (In manuscript.) Presentation at National Meeting of Institute of Food Technologists, Detroit, Mich., 1952. Subtilin when mixed with pea puree gave significant reductions of the thermal death times for Cl. botulinum 62A and the putrefactive anaerobe 3679. Of the three concentrations tested (equivalent to 3.5, 7, and 14 mg. of pure subtilin per liter) the highest gave the greatest displacements, corresponding to 2.5°F. for Cl. botulinum (in the range 230 to 260°F.) and 5°F. for P.A. 3679 (in the range 240 to 270°F.).

Andersen, A. A., WRRL. Effect of subtilin on spores of Clostridium botulinum. Jour. Bact. 64(2):145-9, Aug. 1952. Eight cultures of Cl. botulinum and the closely related putrefactive anaerobe No. 3679 were sensitive to subtilin. Spores of Cl. botulinum 62A did not produce colonies in pork infusion agar containing 0.4 ppm. subtilin /70 percent pure/ and 0.1 ppm. gave a 50 percent reduction in count. The number of viable spores of this strain fell rapidly in subtilin-containing broth which permitted germination, but only slowly in subtilin-containing broth in which germination was repressed by a deficiency of bicarbonate. (Preliminary announcement in Proc. 50th General Meeting of Society of American Bacteriologists, May, 1950, pp. 28-29.)

Andersen, A. A., Michener, H. D., and Olcott, H. S., WRRL. Effect of some antibiotics on Clostridium botulinum. (In manuscript.) Of 28 antibiotics tested, 11 were active in preventing growth of spores of Cl. botulinum 62A in agar at 20 ppm. or less; among the most active were penicillin G, terramycin, aureomycin, and subtilin.

Michener, H. D., WRRL. The action of subtilin and heat on bacterial spores. (In manuscript.) The presence of subtilin increased the thermal death rate (85°C.) of spores of 10 species of Bacillus and one of Clostridium. At incubation temperatures subtilin was found to have neither a sporostatic nor a sporicidal action; from phase microscopic and other observations it was concluded that subtilin killed spores after the process of germination had begun. (Preliminary announcement in Proc. 50th General Meeting of Society of American Bacteriologists, May, 1950, pp. 28-29.)

Michener, H. D., WRRL. The bactericidal action of subtilin on Bacillus stearothermophilus. (In manuscript.) B. stearothermophilus FS 1518 was found to comprise two components, one of which produced rough colonies and was killed by 5 ppm. of subtilin [70 percent pure], the other of which produced smooth colonies and tended to remain dormant except immediately after heat activation.

PHARMACOLOGY

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--Antibiotic produced by Bacillus subtilis. II. Toxicity of subtilin to living embryonic tissue. Proc. Soc. Expt. Biol. and Med. 61:23-24, 1946. Partially purified subtilin possessed a low toxicity for embryonic chick heart tissue cultures. Exposure to a 0.2 percent solution for 10 minutes at 37°C. was required to kill the cultures. A concentration of 0.01 percent was required to kill Staphylococcus aureus under similar conditions.

Anderson, H. H., Villela, G. G., Hansen, E. L., and Reed, R. K., University of California Medical School, San Francisco. Some physical and biologic properties of subtilin and other antibiotics. Science 103(2675):419-20, 1946. Partially purified subtilin proved active in vitro against Lactobacillus plantarum, Endamoeba histolytica and its associated bacterium 't', and Trypansoma equiperdum, but not against Ascaris suis or Leishmania donovani (also tested in vivo). The concentrations required for antibiotic effect also lowered surface tension appreciably. Intravenous injection of 1 percent solution in mice gave an LD_{50} of 60 ± 3 mg. per kg.; subcutaneous injection gave LD_{50} of 670 ± 30 mg. per kg. When given intragastrically, 5.0 grams/kg. killed. One percent solution instilled into the rabbit's eye was nonirritating.

Chin, Y. C., University of California Medical School, San Francisco. Biologic properties of subtilin in physiologic saline solution. Fed. Amer. Socs. Expt. Biol. Proc. 6(1):317, 1947. The toxicity of partially purified subtilin was reduced without loss of bacteriostatic or amebacidal potency by extracting with 0.9 percent sodium chloride solution. The extract contained the antimicrobial activity.

Wilson, R. H., Humphreys, E. M., Reynolds, D. M., and Lewis, J. C. Pharmacology Laboratory and WRRL. Absorption of subtilin in the rabbit. Proc. Soc. Expt. Biol. Med. 71:700-705, 1949. Subtilin [80 to 90 percent pure] had a very low toxicity when administered subcutaneously; 3 gm. per kg. of body weight did not kill mice. Because of its low solubility in physiological saline or serum, subtilin administered subcutaneously or intramuscularly precipitates at the site of injection; blood levels ranging from a trace to 1.4 ppm. were obtained from a dose of 100 mg. per kg. A sterile abcess was still present in muscles 2 months after subtilin injection. Intraperitoneal and oral administration gave no higher levels. Two to 4 ppm. was found in the blood after rectal administration. Intravenous administration gave blood levels of 100 to 200 ppm. of subtilin, which fell to 10 to 30 ppm. in 2 hrs. and to zero in 24 hrs. As much as 7 percent of the subtilin was found in the urine collected within 2 hours. High levels were maintained for 4 hrs. by intravenous infesion without apparent harm. Subtilin methyl esters (5.9 and 11.9 equivalents of OCH_3 per 10^4 gm.) gave no higher blood levels than did subtilin. Sensitive bacteria for the cup-plate assay of subtilin are mentioned (20 mm. zones with 1 to 2 ppm.). (Preliminary announcement in Fed. Amer. Socs. Expt. Biol. Proc. 7(1):266, 1948.)

Wilson, R. H., DeEds, F., and Rather, L. J., WRRL and Stanford Univ. School of Medicine. Toxicity studies on subtilin. Proc. Soc. Expt. Biol. Med. 78:517-519, 1951. Subtilin was fed to rats at levels up to 0.1 percent of the diet for 220 days. No detrimental effects were found on growth, activity, and organ weights, or by macroscopic and microscopic examination of the tissues.

THERAPEUTIC ACTIVITY

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--Antibiotic produced by Bacillus subtilis. III. Effect on type III pneumococcus in mice. Proc. Soc. Expt. Biol. and Med. 62:40-42, 1946. Partially purified subtilin in repeated doses of 0.05 mg. injected intraperitoneally quickly cured experimental type III pneumococcus infections induced in mice by intraperitoneal inoculation, without apparent toxic reaction in the animals. A group untreated until 9 hours after infection were in very bad condition before treatment, but appeared almost normal after 2 or 3 injections.

Salla, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--Antibiotic produced by Bacillus subtilis. IV. Effect of subtilin on the course of experimental anthrax infections in guinea pigs. Proc. Soc. Expt. Biol. and Med. 63:41-42, 1946. Partially purified subtilin in doses of 6 mg. injected intraperitoneally at frequent intervals over a period of 11 days protected guinea pigs from experimental anthrax infections induced by intraperitoneal inoculation, without apparent toxic reaction in the animals. Initial treatments made 3, 6, and 9 hours after inoculation. Untreated controls died in about 3 days.

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--Antibiotic produced by Bacillus subtilis. V. Effect on Streptococcus pyogenes infections in mice. Proc. Soc. Expt. Biol. and Med. 63:519-20, 1946. Partially purified subtilin in 0.1 or 0.2 mg. repeated doses injected intraperitoneally quickly cured experimental Streptococcus pyogenes infections induced in mice by intraperitoneal inoculation, without apparent toxic reaction in the animals. Animals first given subtilin 9 hours after inoculation were in an advanced stage of disease but recovered promptly.

Salle, A. J., University of California at Los Angeles. The nature, properties, and toxicity of subtilin, and its chemotherapeutic effect on the course of experimental infections in animals. Presentation at the Conference on Antibiotic Research, Washington, D.C., Jan. 31-Feb. 1, 1947, under the auspices of the Antibiotics Study Section of National Institute of Health. A review of AIC-106 (see page 3) and of published (see Salle and Jann, above) and unpublished original work. Partially purified subtilin in twice repeated 1 mg. doses injected intraperitoneally quickly cured intraperitoneally induced Staphylococcus aureus infections in mice. Definite suppressive action on experimental tuberculosis infections in guinea pigs was claimed (see below).

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Preparation of a modified subtilin suitable for the treatment of tuberculosis and other infections in animals. Jour. Clin. Invest. 28(5):1036-38, 1949. A solution of partially purified subtilin in 10 percent urea, from which part of the impurities are believed to be removed by addition of pectin, is stated not to produce nodule-like deposits when injected subcutaneously in guinea pigs. Tuberculosis-infected guinea pigs treated for 6 weeks with a total of 22 mg. of subtilin per day (in three subcutaneous injections) showed relatively few lesions in liver, lungs, and spleen when one lot of subtilin was used, but relatively little tuberculostatic effect was noted when another lot of subtilin was used. Animals treated simultaneously with subtilin and with a sub-optimal dosage of streptomycin showed much less tuberculous involvement than with either substance alone.

Anderson, H. H., and Wong, S. C., University of California Medical School, San Francisco. Antibiotics in experimental tuberculosis. Tuberculology 8(3):77-82, 1946. Partially purified subtilin at 2.5 ppm. prevented growth of virulent strains of Mycobacterium tuberculosis in a modified Dubos medium. The partially purified preparations used gave a marked anaphylactic reaction with guinea pigs. Topical application of subtilin was ineffective in treating experimentally induced tuberculous infections of the cornea of rabbits. Daily subcutaneous injections of 6 mg. of subtilin continued over a period of 6 weeks failed to affect the course of experimentally induced tuberculosis in Syrian hamsters. Therapy was initiated 8 days after infection. One particular lot of subtilin was well tolerated; two others gave marked local tissue toxicity. It was believed that failure of therapy was related to precipitation by physiological concentrations of NaCl. Such concentrations reduced the in vitro antibiotic activity of subtilin on Lactobacillus planatarum.

Farber, S. M., Eagle, H. R., Anderson, H. H., and Gorman, R. D., University of California Medical School, San Francisco. Topical application of subtilin to tuberculous lesions in man. Jour. Lab. Clin. Med. 33(7):799-804, 1948. Subtilin solutions were administered by inhalation to 8 tuberculous patients. Amounts ranging from 4 to 50 gm. were used for periods of 2.5 to 10 months. Suggestive evidence of therapeutic activity was observed in 3 cases. No evidence of serious toxicity was observed.

Steenkin, W., and Wolinsky, E., Trudeau Laboratory, Trudeau, New York. The tuberculostatic effect of subtilin in vitro and in vivo. Jour. Bact. 57(4):453-57, 1949. Partially purified subtilin inhibited the $H37R_v$ strain of tubercle bacillus when used in moderate concentrations (10 to 100 ppm.) in medium containing sorbitan monooleate, but not in other media lacking sorbitan monooleate which gave granular growth of the bacillus. Tuberculous guinea pigs treated intramuscularly with 1 to 2 mg. of subtilin every 12 hrs. for 64 days exhibited as much gross tuberculous disease on autopsy as did the controls. Four mg. of this lot of partially purified subtilin per animal of 400 to 600 gm. per day was near the maximum tolerated dose, since progressive weight losses were observed.

Eagle, H., Musselman, A. D., and Fleischman, R., United States Public Health Service. The action of bacitracin and subtilin on Treponema pallidum in vitro and in vivo. Jour. Bact. 55(3):347-58, 1948. Subtilin proved about one-half as active as penicillin G against Streptococcus pyogenes (C-203), 0.03 ppm. inhibiting in vitro. Against T. pallidum (Reiter strain) subtilin was approximately 0.01 as active in vitro as penicillin G, 2 to 4 ppm. being necessary for bacteriocidal action. Subtilin at 10 mg. per kg. administered intramuscularly 4 times daily for 4 days failed to eliminate the treponemata from the testicular chancres in rabbits.

Courtillet, D. Contribution a l'etude des substances antibiotiques produites par les bacteries du groupe du *Bacillus subtilis*. Imprimerie Richard, Paris, 1949, 125 pp. An extensive review of the antibiotic activities reported for the species *B. subtilis*, with special reference to subtilin. Original observations are reported on production, extraction, assay, stability, antibiotic spectra, toxicity, and activity against *Diplococcus pneumoniae*, *Streptococcus hemolyticus*, and *Staphylococcus aureus* in mice, which are in general agreement with the results of the American authors. Of special interest are the observations that high concentrations (1 percent) of cysteine antagonized the action of subtilin on *D. pneumoniae*; that subtilin was inactivated by papain; that the action of subtilin on growth of *S. aureus* was one of increasing the lag phase without decreasing the subsequent rate of multiplication; and that subtilin exhibits relatively narrow bacteriostatic:bactericidal concentration ratios (1:20 to 1:160) for various bacteria. It was noted that the simultaneous administration of subtilin and polyvinylpyrollidone (Subtosan) gave better protection against delayed infection of mice with *S. hemolyticus* than did subtilin alone, although the minimal protective appeared to be increased.

Heishman, J. O., Bur. of Animal Industry, USDA, Beltsville, Md. Report on subtilin and bacitracin as possible treatment for bovine mastitis. *Amer. Jour. Vet. Res.* *11*, 206-10, 1950. Although subtilin was active *in vitro* against micro-organisms associated with bovine mastitis, subtilin in amounts up to 0.4 gm. per quarter in two doses caused elimination of the infection from only two of 15 quarters infected with *Streptococcus agalactiae*, and had no permanent effect on the infection with *Str. disgalactiae* (3 quarters) or with hemolytic Staphylococcus (4 quarters).

Carminati, G. M., and Ercoli, N., Instituto Sieroterapico Milanese Serafino Belfanti. Azione immediate e curativa di alcuni antibiotici nell'infezione sperimentale con spirochete della febbre ricorrente. *Bollettino I.S.M.* 30(2):97-104, 1951. Subtilin showed a marked activity against relapsing fever in mice; quantitatively its activity was much higher than that of penicillin G against *Borrelia novyi* and of the same magnitude as that of penicillin against *B. duttoni*. A sterilizing effect was obtained following single doses of 20 to 25 mg. of subtilin per kg. of body weight.

FOOD PRESERVATION

Andersen, A. A., and Michener, H. D., WRRL. Preservation of foods with antibiotics. I. The complementary action of subtilin and mild heat. *Food Technol.* 4(5):188-89, 1950. The combination of 5 to 10 ppm. of subtilin [30 to 90 percent pure] and mild heat treatment (100°C. for 5 or 10 minutes) served to protect canned vegetables against microbial spoilage (for periods up to 106 days) as well as the sterilization under steam pressure required by conventional practice. Peas, asparagus, and corn have been processed successfully by this technique. The asparagus was inoculated with *Bacillus stearothermophilus*, a thermophile which frequently causes loss in commercial practice.

Tarr, H.L.A., Southcott, B. A., and Bissett, H. M., Fisheries Research Board, Canada. Effect of several antibiotics and food preservatives in retarding bacterial spoilage of fish. *Progress Repts.* *83*, 35, 1950. Subtilin (50 ppm.) was valueless in this connection, whereas aureomycin and terramycin showed some promise in preliminary experiments.

Adams, A. T., Ayres, J. C., and Tischer, R. G., Iowa State College, Ames, Iowa. The effect of subtilin and heat in preventing spoilage of comminuted beef. Food Technol. 5(2):82, 1951. Subtilin at high concentrations (100 or 1000 ppm.) 73 percent potency when heated with comminuted beef inoculated with 100 or 10,000 spores of putrefactive anaerobe No. 3679 per gm. delayed but did not prevent spoilate. (Preliminary announcement in Proc. 51st General Meeting of Society of American Bacteriologists, May, 1950, p. 16.)

Burroughs, J. D., and Wheaton, I. E., American Can Co. Studies on the preservative action of antibiotics in processed foods. Canner 112(10):50-55, 1951. The presence of 40 or 80 ppm. of partially purified subtilin in peas processed at 212°F. for 35 min. prevented spoilate for 3 months in the absence of artificial inoculation, and delayed spoilage when the peas were inoculated with 500,000 to 1,000,000 spores of a mixture of 5 Type A and 5 Type B strains of Clostridium botulinum. Of gramicidin (100 ppm.) with peas, methylol-gramicidin (100 ppm.), bacitracin (40 ppm.), and streptomycin (40 ppm.) in corn, only the latter partially prevented spoilage, and that only with an uninoculated pack. On the other hand subtilin (40 ppm.) prevented spoilage (2 months) of tomato juice, whether or not it was inoculated with 12,000 spores of Bacillus thermoacidurans per ml. Although 350 spores per ml. survived processing, none were detected after 30 days of incubation.

Denny, C. B., Bohrer, C. W., and Cameron, E. J., National Canners Assoc.,Washington, D.C. N.C.A. experimental program with antibiotics. Research Report No. 7-51, June, 1951. A report of laboratory investigations and experimental packs with antibiotics, with particular reference to subtilin. Mesophilic bacteria, particularly the putrefactive anaerobes which include Clostridium botulinum, were most resistant to subtilin-mild heat treatment whereas the strongly heat-resistant thermophiles, particularly the flat sour types, were most sensitive. Subtilin-mild heat treatment generally failed to maintain the preservation of inoculated packs of a number of food products, whereas 20 ppm. of partially purified subtilin in combination with mild heat appeared sufficient to prevent spoilage except with broccoli and celery. Flat sour spoilage in heavily inoculated tomato juice did not occur even after extended incubation. Spoilage in packs inoculated with mesophilic putrefactive anaerobic types including Cl. botulinum, although delayed, was practically complete. In quality comparisons, subtilin packs were much brighter, firmer, and clearer than were the regularly processed packs.

Cameron, E. J., National Canners Assoc., Washington, D.C. Use of antibiotics in preserving foods. Proc. Third Conference on Research of the American Meat Institute, pp. 32-38, 1951. A review.

Cameron, E. J., and Bohrer, C. W., National Canners Assoc., Washington, D. C. Food preservation with antibiotics: The problem of proof. Food Technol. 5(8):340-42, 1951. The elements of proof necessary to establish the acceptability of an antibiotic in food preservation are considered to include laboratory and pilot-scale demonstration of destruction of spores of Clostridium botulinum and other spoilage organisms. Examples showing that subtilin does not meet this criterion are offered.

Godkin, W. J., and Cathcart, W. H., The Great Atlantic and Pacific Tea Co., New York. Effect of antibiotics in retarding the growth of Micrococcus pyogenes var. aureus in custard fillings. Food Technol. 6, 224-29, 1952. Subtilin (10 to 20 ppm.) /70 percent pure/, penicillin (1.2 ppm.), terramycin (0.4 to 1 ppm.), and aureomycin (0.6 to 1 ppm.) were effective in retarding for 24 hrs. the outgrowth of M. pyogenes var. aureus inoculated into autoclaved custard fillings, whereas bacitracin (60 ppm.), chloromycetin (5 ppm.), and streptomycin (5 ppm.) were ineffective. On the other hand only subtilin (50 or 100 ppm.) was effective alone in retarding both the heat-resistant natural spoilage flora and the inoculated food pathogen in non-autoclaved filling. A combination of subtilin (100 ppm.) and terramycin (1 ppm.) was highly effective with the inoculated non-autoclaved filling and it was considered that it would protect custard fillings at room temperature for 3 days.

RELATED COMPOUNDS

Michener, H. D., and Snell, N., WRRL. Two antifungal substances from Bacillus subtilis cultures. Arch. Biochem. 22(2):208-14, 1949. Cultures of B. subtilis ATCC No. 6633 possessed antifungal activity which was not exhibited by partially purified subtilin preparations. A single extraction with butanol at pH 2.5 removed most of the activity against Aspergillus niger but not against Rhizopus solani, thus indicating that at least two antifungal substances were present. Concentration procedures are given.

Walton, R. B., and Woodruff, H. B., Merck & Co., Rahway, New Jersey. A crystalline antifungal agent, mycosubtilin, isolated from subtilin broth. Jour. Clin. Invest. 28(5):924-26, 1949. B. subtilis ATCC No. 6633 broth yielded white crystalsof "mycosubtilin", melting point 256-257°C., insoluble in all reagents tested except in pyridine, warm 70 percent ethanol, and dilute NaOH. The acid hydrolyzate when assayed microbiologically yielded 45 percent of aspartic acid, 5.4 percent of tyrosine, and 4.2 percent of proline. Mycosubtilin is heat stable. 1.5 ppm. inhibits the growth of a Trichophyton strain (MF301) on agar, although 200 ppm. of subtilin is inactive. The action is neutralized by the addition of 10 percent of horse serum. Mycosubtilin inhibits the growth of many but not all filamentous fungi and yeasts at 1.5 to 20 ppm.